

7a. X NO 7b. Assurance of Compliance # 8. VERTERRATE ANIMALS 8a. NO X YES ... IACUC Approval Date TELEPHONE INFORMATION 8b. Animal Welfare Assurance # A3392-01 12a, PRINCIPAL INVESTIGATOR AREA TELEPHONE NO. CODE AND EXTENSION 9. PERFORMANCE SITE(S) (organizations and addresses) PROGRAM DIRECTOR (Item 2a) Indiana University School Byoung S. Kwon 317 274-3965 of Medicine 125, NAME OF BUSINESS OFFICIAL Dept. Microbiology & Immunology (Item 6) 635 Barnhill Drive William E. Farguhar 812 855-3962 Indianapolis, IN 46241 12c, NAME AND TITLE OF OFFICIAL SIGNING FOR APPLICANT ORGANIZATION (Item 15) Wendell F. McBurnev 317 274-8285

13. USE THIS SPACE FOR CORRECTIONS TO ITEMS 1 THROUGH 6. INDICATE THE NUMBER(S) WHERE ANSWERS APPLY.

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application. Willivij provision of false information is a criminal offense. (U.S. Code, Title 13, Section 1007).	(In ink. "Per" signature not acceptable) Appurffe fivor	DATE
15. CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true and complete to the bast of my knowledge, and accept the obligation to comply with the Public Health Service terms and conditions if a grant is awarded as the result of this application. A willfully faise certification is a criminal offense. IU.S.	(in ink. Sper signature not acceptable)	DATE

	GET FOR FROM		TH	ROUGH	GRANT	NUMBER	
NEXT BUDGET					A.T.	28175-2	
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PERSONNEL (Applicant of		7		EFFORT	DOLLAN AM	FRINGE	I Committee
NAME NAME	ROLE IN PROJECT	TYPE APPT.	45 OF APPT.	PROJ.	SALARY	BENEFITS	TOTAL
Byoung S. Kwon	Principal investigator	1.0	46	0.46	28,130	9,522	37,65
Karen Z. Pollok	Postdoctoral Fellow	1.0	100	1.0	20,000	4,980	24,98
Hal E. Broxmeyer	Co-Investigator	1.0	3	0.03	2,388	808	3,19
Kwi 0. Oh	Co-Investigator	1.0	100	1.0	13,532	3,370	16,90
Yvonne Kobayashi	Research Tech.	1.0	100	1.0	20,168	5,023	25,19
Zhen Zhou	Scientist	1.0	30	0.3	4,400	1,096	5,49
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SUPPLIES (Itemize by category)							
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GRANT NUMBER

SECTION I (continued)
NEXT BUDGET PERIOD

AI 28175-02

B. SUPPLEMENTAL INFORMATION REGARDING ITEMS IN THE PROPOSED BUDGET FOR THE NEXT PERIOD WHICH REQUIRE EXPLANATION OR JUSTIFICATION. (SEE INSTRUCTIONS)

Personnel

<u>Dr. Byoung S. Kwon</u>, Principal Investigator (46% effort), is responsible for the overall conduct of this research. His efforts include performance of daily work at the laboratory bench; coordination of work with the collaborating laboratories of Drs. Hal Broxmeyer, Leonard Schultz, Sherman Weissman, and John Ding-E Young; administration of the project budget, and writing of annual reports. Dr. Kwon will design and construct expression plasmids, and perform receptor-binding studies and functional assays, evaluating each experimental step in the program.

<u>Dr. Karen Pollok,</u> (100% effort) is a Postdoctoral fellow in this laboratory. Her responsibilities will include protein purification of soluble 4-lBB and perforin and a variety of immunological assays. Dr. Pollok will perform a number of

biochemical and receptor-binding assays to characterize 4-1BB molecule.

Dr. Hal Broxmeyer, Coinvestigator (3% effort), participates in evaluating functions of recombinant products of L2G25B and L2G25C in both in vitro and in vivo.

Dr. Kwi-Ok Oh, Coinvestigator (100%), a Visiting Scientist, has been receiving training in this laboratory since. She will maintain cell culture, transfect recombinant DNA and perform protein purification and receptor-binding assaws L2025B and L2025C proteins under the instruction of Dr. Kwon.

Miss Yvonne Kobayashi, (100%) Research Technician, will prepare DNAs, perform Southern, Northern, and Western blotting procedures, and screen hybridomas by dotimmunoassay. Yvonne is well-trained in DNA-sequencing. She is also well trained in handling vaculovirus expression systems. She has been producing recombinant proteins for L2625C, perforin and 4-DB. Yvonne will also be involved in purification of a large quantity of perforin to crystalize.

Dr. Zhen Zhou, (30%) Visiting Scientist, will perform immunocytochemistry to localize the 4-1BB proteins in various mouse organs. He is also doing in situ hybridization with the gene probes we generated. Dr. Zhou will also be involved in the studies on 4-1BB and perforin expression in the T cells infiltrated in autoimmune insulfits and arthritis of mice.

Consultants \$600

The amount of \$600 is requested to defray travel expenses of Dr. Kwon for the purpose of conferring once each year with Drs. Weissman and Shultz. No costincrease is projected in subsequent grant-years for this consultant expense.

 $\underline{\text{Dr. Sherman Weissman}}$ will continue to provide his expertise in nucleic acid research in all phases of this project. He advises on production of recombinant proteins in both prokaryotic and eukaryotic expression systems. He also evaluates the work of this laboratory as to scientific merit.

 $\frac{\text{Dr. John D.-E Young}}{\text{consults with Dr. Kwon on protein purification and assays on CTL activity.}}$

<u>Dr. Leonard Shultz</u>, of Jackson Laboratories, provides immunodeficient mutants collected by him and also provides new mutants as they arise.

<u>Dr. Stephen Litwin</u> consults with this laboratory on B-cell maturation, immunoglobulin production, and assays on B-cell functions.

<u>Dr. Himadri Samanta</u> provides his expertise on the production of recombinant DNAs using the bovine papilloma viral expression system. He also advises on the technologies for purification of the recombinant proteins.

_____KWON000058

<u>Supplies</u> \$23,280

Items in the supply budget remain the same as in grant-year 2, and a $6\mathbb{X}$ increment is applied to allow for increases in costs.

Travel \$1,200

Dr. Kwon and Dr. Pollok plan to attend FASEB meeting.

Other Expenses \$1,600

Costs of publication (page charges, artwork, etc.), computer costs, and mailing/long-distance telephone expenses remain the same as in year 2.

SECTION II	FROM	THROUGH	GRANT NUMBER
CURRENT BUDGET PERIOD			
AND KEY PERSONNEL			AI 28175-0

The following pertains to your CURRENT PHS budget. This information will be used in determining the amount of support for the NEXT

budget period.			
A. CURRENT BUDGET	TOTAL ESTIMATED EXPENDITURES AND OBLIGATIONS (1)	ESTIMATED UNOBLIGATED BALANCE (2)	EXPLAIN ANY SIGNIFICANT ESTIMATED UNOBLIGATED BALANCE IN COLUMN 2 (3)
TOTAL DIRECT COSTS	99,493	99,493	
INDIRECT COSTS (As provided)	48,105	48,105	
TOTALS>	147,598	147,598	

B. CURRENT BUDGET PERIOD KEY PERSONNEL ENGAGED ON PROJECT (Only if different)

NAME, DEGREE(S) SSN	POSITION TITLE AND ROLE IN PROJECT DEPARTMENT AND ORGANIZATION	CHANGE IN % OF EFFORT
Karen Z. Pollok, Ph.D. 231-84-3958	Postdoctoral Fellow	+ 1.0
Yvonne Kobayashi, B.S. 306-64-6558	Lab Technician	+ 1.0
Zhen Zhou, M.D.	Visiting Scientist	+ 0.3

C. and D. (Only if different)

See instructions and provide the information required in Items C. and D. Use this page and continuation pages as necessary.

See Attached

THE REPORT OF THE PROPERTY OF

NAME, DEGREE(S), SSN	POSITION TITLE AND ROLE IN PROJECT	DEPARTMENT AND ORGANIZATION
Karen Z. Pollok, Ph.D.	Postdoctoral Fellow	Microbiology and Immunology Indiana University School of Medicine
Yvonne Kobayashi, B.S.	Lab Technician	Microbiology and Immunology Indiana University School of Medicine
Zhen Zhou, M.D.	Visiting Scientist	Microbiology and Immunology Indiana University School of Medicine

C and D (continued)

- C. <u>Equipment</u> Other funds were identified to purchase the requested Micro centrifuge. No other equipment was purchased.
- D. Travel The following trips were supported:
 - B.S. Kwon to Berlin, W. Germany -7th International Congress of Immunology - \$1,242 Presented a poster.
 - 2) B.S. Kwon New Orleans, LA \$727 ASBMB/AAI Joint Meeting -Spoke at a mini-symposium and presented a poster.
 - 3) Kack Kim Hilton Head, SC \$460 Second International Workshop on cytokines. Presented a poster. Dr. Kim's salary is not supported by this grant but he is studying perforin gene expression, a part of this project. Dr. Kim is Dr. Oh's husband.
 - 4) Dr. Oh Hilton Head, SC \$257
 Second International Workshop on cytokines.
 Presented a poster.

OTHER SUPPORT

(Use continuation pages if necessary)

GRANT NUMBER

AI 28175-02

FOLLOW INSTRUCTIONS CAREFULLY. Incomplete, inaccurate, or ambiguous information about OTHER SUPPORT could lead to delays in the award. OTHER SUPPORT to be listed here refers to all current or requested support whether related to this application or not. If there are changes subsequent to submission, notify the Grants Management Official named on the Notice of Grant Award.

For each of the key personnel named on page 4, list, in three separate groups: (1) all currently active support; (2) all applications and proposals pending review or funding; and (3) applications and proposals planned or being prepared for submission. Include all Federal, non-Federal (e.g., for-profit, pharmaceutical, foundations), and institutional research, training, and other grant, contract, and fellowship support at the applicant organization and elsewhere. If part of a larger project, identify the principal investigator/program director and provide the data for both the parent project and the subproject. If none, state "none."

For each item give: (a) the source of support, identifying number and title; (b) percentage of appointment on the project; (c) dates of entire project period; (d) annual direct costs; (e) a brief description of the project; (f) whether the item overlaps, duplicates, or is being replaced or supplemented by the present application, delineate and justify the nature and extent of any scientific and/or budgetary overlaps or boundaries; and (g) any modifications that will be made should this continuation award be made.

PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR:

(1) CURRENTLY ACTIVE SUPPORT: (a)

- a) NIH Grant 1R01 AR40248-01; Melanin Biosynthesis and Oculocutaneous Albinism b) B.S. Kwon, Principal Investigator. 10% effort. c)
- d) Annual cost \$128,705 e) Studies on the molecules involved in melanin pigment synthesis. f) Not overlap with current project.
- a) Schering-Plough Corporation; Regulation of Melanin Biosynthesis (Chemicals)
- b)B.S. Kwon, Principal Investigator, 10% effort, c)
- d) Annual cost \$140,500 e) Induction of darkening and lightening of skin pigment by chemical agents. f) Not overlap with current project.
- a) March of Dimes Birth Defects Foundation; Molecular Basis of tyrosinase-negative albinism b) B.S. Kwon, Principal Investigator. 5% effort. c)
- d) Annual direct cost \$35,000 e) Molecular genetic studies on the mutation responsible for tyrosinase-negative albinism. f) Not overlap with current project.
- a) Diabetes Research and Training Center Pilot Project; Role of T-lymphocytes in the pathogenesis of IDDM b) B.S. Kwon, Principal Investigator. 5% effort. c) d) Annual direct cost \$24,000 e) Studies on the genes expressed in the infiltrating T-cells in insulitis. f) Not overlap with current project.

KWON000062

PHS 2590

GRANT NUMBER		
AI 28175-	AI 28175-02	
PERIOD CO	VERED BY THIS REPORT	
FROM	THROUGH	
	AI 28175- PERIOD CO	

Characterization of three new T_lymphocyte-specific gene (SEE INSTRUCTIONS)

1. The main objective for the forthcoming project year is to determine the functions of L2G25B, L2G25C (formerly called L2G95B) and 4-1BB. We will also study the domain-specific function of perforin. We first prepared antibodies against oligopeptides representing the deduced primary sequence of each molecule and then prepared recombinant proteins of these molecules. The following is a brief summary of unpublished results and future plans.

A. 4-1BB:

4-IBB protein is a receptor molecule which is inducible in T cells and is expressed constitutively in neurons and in certain medulla cells in kidney. Cytoplasmic domain of 4-IBB contains consensus sequence (-C-R-C-P-) for the binding to p561ck, a protein tyrosine kinase. We are preparing soluble form of the 4-IBB, which is recombinant protein without the transmembrane domain. This can be a specific inhibitor to the 4-IBB, as shown in other lymphokine receptors. We have expressed 4-IBB in Cl27 cells using bovine papilloma viral vector and in insect cells (sf-9) using baculoviral vector. These material will be used for two purposes; 1) to find the potential ligand; and 2) to determine whether 4-IBB transduces signal through binding to protein tyrosine kinases such as p561ck.

B. Perforin:

Perforin is believed to be a key molecule in killing cells bearing foreign antigens by forming a transmembrane channel. We isolated and characterized the cDNA and a gene for mouse perforin. We have been successful in producing the recombinant perforin (both human and mouse) in a baculoviral expression system. The purpose is to crystalize the molecule to understand its three dimensional structure. This information in turn will be used to design experiment to determine the domain-specific functions of this molecule.

C. L2G25B and L2G25C:

We have prepared a large quantity of recombinant proteins for both genes. According to in vitro experiments done in collaboration with Dr. Hal Broxmeyer the L2G25B and L2G25C recombinant proteins have direct myelopoietic enhancing activity for mature progenitors, while L2G25B, but not L2G25C, has direct suppressing activity for more immature progenitors. We plan to determine the physiological relevance of the myelopoietic enhancing and suppressing activities of these molecules in animal models. We are also seeking cells bearing receptors to the L2G25B and L2G25C proteins. The cells that we are employing at present are FDCP-Mix, 416B, and DUS2B, which represent bone marrow stem cell lines; I1-1-activated endothelial cells; activated spleen cells; and primary bone marrow cell mixture. We hope this study leads to finding the target cells through which we can study in depth the functions of these small molecules.

- 2. Our main effort was directed to prepare reliable reagents to utilize for the determination of functions of T-cell molecules, 4-lBB, L2G25B, L2G25C and perforin. All the molecules have been produced in recombinant form and antibodies recognizing the natural molecules are prepared. In addition to the preparative work mentioned above, we found followings in each molecule. 1) 4-lBB was expressed not only in T-cells but also in neuron and kidney cells. 4-lBB was expressed in early stage of insulitis but not in later stage of insulitis in NOD mice. 2) 5' UTR (untranslated region) of mouse perforin is formed by alternative splicing. The perforin mRNA was degraded within 5 min. when the killer cells (CTL, NK and LAK) made a full contact with specific target. Mouse perforin gene was characterized and mapped to chromosome #10. 3) L2G25B and L2G25C recombinant proteins have direct myelopoletic enhancing activity for mature progenitors, while L2G25B, but not L2G25C, has direct suppressing activity for more immature progenitors. Our recombinant proteins for L2G25B and L2G25C were as active as natural ones.
- 3. and 4. Non-applicable.
- 5. Seven papers have been published or are now in-press and one is in submission during the second grant-year: Copies of those papers are attached.
- Kwon, B.S., Kestler, D.P., Eshhar, Z., Oh, K.-O. and Wakulchik, M. Expression characteristics of two potential T cell mediator genes. <u>Gellular Immunology</u> 121: 414-422, 1989.
- Qureshi, M., Yoon, J.W. and Kwon, B.S. Identification and production of monoclonal antibodies against a discriminating protein molecule between B and D variants of encephalomyocarditis virus. Develop. Comp. Immunol. 13: 263-271, 1989.
- Liu, C.C., Joag, S.V., Kwon, B.S., and Young, J.D-E. Induction of perforin and serine esterases in murine cytotoxic T lymphocyte clone. <u>J. Immunol</u>. 144: 1196-1201, 1990.
- Joag, S.V., Liu, C.C., Kwon, B.S., Duke, R.C., Clark, W.R., and Young, J.D.-E. The expression of pore-forming protein and two serine esterase genes in murine primary and cloned effector lymphocytes. J. Cell. Biochem. 43: 81-88, 1990.
- Trapani, J.A., Kwon, B.S., Kozak, C.A., Chintamaneni, C., Young, J. D-Z and Dupont, B. Genomic organization of the mouse pore-forming protein (perforin) gene and localization to chromosome 10. J. Exp. Med. 171:545-557, 1990.
- Young, J.D.-E., Kwon, B.S., Trapani, J.A., Liu, C.-C. and Young, L.H. Lymphocyte-mediated cytolysis: role of granule mediators. Subcellular Biochem. (in press).
- Broxmeyer, H.E., Sherry, B., Cooper, S., Oh, K., Tekamp-Olson, P., Kwon, B.S., and Cerami, A. Enhancing and suppressing effects of recombinant murine macrophage inflammatory proteins on colony formation in vitro by bone marrow myeloid progenitor cells. Blood. (in press).
- Bajapi, A., Kwon, B.S. and Brahmi, Z. Rapid loss of perforin and serine protease RNAs in cytotoxic lymphocytes exposed to sensitive targets. <u>J. Immunol</u>. (submitted)

(1)	
	GRANT NUMBER
CHECKUST	AI 28175-02
Check the appropriate boxes and provide the information requested. Make this pag Do not attach copies of this page to the duplicated copies of the application.	e the last page of the signed original of the application
ASSURANCES	
The following certifications described below are made by checking the appropriate box SIGNING FOR APPLICANT ORGANIZATION on the FACE PAGE of the application.	es and verified by the signature of the OFFICIAL
a. Delinquent Federal Debt X No Yes (If "Yes," attach explanation.)	
Before a grant award can be made, the applicant organization must certify that it is in The certification applies to the applicant organization, not to the person signing the principal investigator/program director.	application as the authorized representative nor to the
Examples of Federal debt include delinquent taxes, audit disallowances, guaranteed other miscellaneous administrative debts. For purposes of this certification, the foll	or direct student loans, FHA loans, business loans, and owing definitions of "delinquency" apply:
For direct loans and fellowships (whether awarded directly to the applicant by th funds), a debt more than 31 days past due on a scheduled payment. (Definition Service Award.)	e Federal Government or by an institution using Federal n <u>excludes</u> "service" payback under a National Research
For guaranteed and insured loans, recipients of a loan guaranteed by the Federa repurchased from a lender because the borrower breached the loan agreement a	I Government that the Federal Government has and is in default.
For grants, organizations in receipt of a "Notice of Grants Cost Disallowance" window not resolved the disallowance. (Definition excludes disallowances in an "a	hich have not repaid the disallowed amount or which ppeal" status.)
Where the applicant discloses delinquency on debt to the Federal Government, the determining whether the prospective grantee organization is responsible with respect until payment is made or satisfactory arrangements are made with the agency to who for the PHS to contact the applicant before a grant can be made to confirm the stat for its liquidation. Applicants that fail to liquidate indebtedness to the Federal Governisk of not receiving financial assistance from the PHS.	PHS shall (1) take such information into account when it to that grant, and (2) consider not making the grant om the debt is owed. Therefore, it may be necessary on the debt and account in the property of the prope
b. Debarment and Suspension. X No Yes (If "Yes," attach explanation)
Before a grant award can be made, the applicant organization must certify, among o debarred, suspended, proposed for debarrent, declared ineligible, or voluntarily ex- department or agency. Subawardees, that is, other corporations, partnerships, or make the same certification to the applicant organization concerning their covered in implementing regulations, Title 45 Odd of Federal Regulations Part 76, for complete	ther things, that neither it nor its principals are presently luded from covered transactions by any Federal ther legal entities (called Tower tier' participants), must anactions. Please refer to the pertinent DHHS certification requirements.
c. Drug-Free Workplace. X Yes No (If "No," attach explanation.)	
Before a grant award can be made, the applicant organization must certify that it will certification require the applicant organization to:	provide a drug-free workplace. The main points of the
Publish a statement notifying employees that the unlawful manufacture, distribution substance is prohibited in the workplace and specifying the actions that will be to approximate the property of the pro	on, dispensation, possession, or use of a controlled aken against employees for violation of such prohibition:
 Establish a drug-free awareness program; Require that each employee engaged in the performance of a grant or contract be 	e provided a copy of the published statement:
 Notify the employee that as a condition of employment, the employee will abide b 	
Notify the PHS awarding component of any employee convicted of a drug violation	
 Require any employee who is convicted of a drug offense occurring in the workpl Please refer to the pertinent DHHS implementing regulations, Title 45 Code of Federa requirements, 	ace to participate in a rehabilitation program. Il Regulations Part 76, for complete certification
INDIRECT COST CALCULATION	
indicate the applicant organization's most recent indirect cost rate established with the i or-profit organizations, the rate established with the appropriate PHS Agency Cost Advi- grants, construction grants, grants to Federal organizations and grants to individuals, and additional instructions provided for Research Career Development Awards, Institutional I grant applications.	appropriate DHHS Regional Office, or, in the case of sory Office. Indirect costs will not be paid on foreign di usually not on conference grants. Follow any Vational Research Service Awards, and specialized
X DHHS Agreement Dated: No Indirect	t Costs Requested
No DHHS Agreement, but rates established with	DATE
*CALCULATION	
Enter proposed budget period:	
Amount of Base \$ 140,097 x Rate Applied 49.0 % Add to total direct costs from page 2 and enter new total	
*Check appropriate box(es)	
Salary and wage base X Modified total direct costs base	Other base (Attach explanation)
Off-site, other special rate, or more than one rate involved (Attach explanation)	KWON00006
PHS 2590 PAGE 7*	Anchoud.
This is the required last page of the application.	